



INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification ⁴ : G01N 33/569, 33/573, C12Q 1/58	A1	(11) International Publication Number: WO 89/ 09407 (43) International Publication Date: 5 October 1989 (05.10.89)
(21) International Application Number: PCT/GB89/00104 (22) International Filing Date: 6 February 1989 (06.02.89) (31) Priority Application Numbers: 8806831 8818531.9 (32) Priority Dates: 23 March 1988 (23.03.88) 4 August 1988 (04.08.88) (33) Priority Country: GB (71)(72) Applicant and Inventor: DENT, Julie, Claire [GB/ GB]; 47 Hillview Road, Ealing, London W13 0HA (GB). (74) Agents: DUNLOP, Brian, Kenneth, Charles et al.; Wynne-Jones, Laine & James, 22 Rodney Road, Cheltenham, Gloucestershire GL50 1JJ (GB).		(81) Designated States: AT, AT (European patent), AU, BB, BE (European patent), BG, BJ (OAPI patent), BR, CF (OAPI patent), CG (OAPI patent), CH, CH (Eu- ropean patent), CM (OAPI patent), DE, DE (Euro- pean patent), DK, FI, FR (European patent), GA (OAPI patent), GB, GB (European patent), HU, IT (European patent), JP, KP, KR, LK, LU, LU (Euro- pean patent), MC, MG, ML (OAPI patent), MR (OA- PI patent), MW, NL, NL (European patent), NO, RO, SD, SE, SE (European patent), SN (OAPI pa- tent), SU, TD (OAPI patent), TG (OAPI patent), US. Published <i>With international search report.</i>
(54) Title: DETECTION OF CAMPYLOBACTER PYLORI UREASE ANTIBODIES AND REAGENT THEREFOR		
(57) Abstract A reagent for use in the diagnosis of <i>Campylobacter pylori</i> infections comprising <i>C.pylori</i> urease attached to a solid surface. Such a reagent may be used in an ELISA test or a latex agglutination test to detect antibodies to <i>C.pylori</i> urease.		

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AT	Austria	FR	France	ML	Mali
AU	Australia	GA	Gabon	MR	Mauritania
BB	Barbados	GB	United Kingdom	MW	Malawi
BE	Belgium	HU	Hungary	NL	Netherlands
BG	Bulgaria	IT	Italy	NO	Norway
BJ	Benin	JP	Japan	RO	Romania
BR	Brazil	KP	Democratic People's Republic of Korea	SD	Sudan
CF	Central African Republic	KR	Republic of Korea	SE	Sweden
CG	Congo	LI	Liechtenstein	SN	Senegal
CH	Switzerland	LK	Sri Lanka	SU	Soviet Union
CM	Cameroon	LU	Luxembourg	TD	Chad
DE	Germany, Federal Republic of	MC	Monaco	TG	Togo
DK	Denmark	MG	Madagascar	US	United States of America
FI	Finland				

Detection of *Campylobacter pylori* urease antibodies and reagent therefor.

The present invention relates to a diagnostic reagent, in particular it relates to a diagnostic reagent for use in the accurate diagnosis of *Campylobacter pylori* infection.

Chronic gastritis is a histological diagnosis based on an increase in the number of chronic inflammatory cells (plasma cells, lymphocytes, histocytes) in the lamina propria of the gastric mucosa. Chronic gastritis is termed active when there is an acute infiltration of neutrophils. Chronic active gastritis is further classified according to its distribution within the stomach. Type A gastritis usually affects acid secreting cells in the stomach in preference to the antrum. This is seen in patients with pernicious anaemia and is believed to be of an auto immune pathogenesis. Type B gastritis primarily affects the pyloric or antral area of the stomach, and is not associated with pernicious anaemia. Furthermore, type B gastritis has been shown to be associated with gastric and duodenal ulcer and occurs with increasing frequency with ageing.

In the past, chronic gastritis has been attributed to many causes including smoking, drugs, and alcohol. Traditionally, duodenal ulceration has been associated with

excess gastric acid secretion, and therefore current surgery and chemotherapy is designed to curtail or reduce this output.

This approach however, may be revolutionised following the culture of a bacterium from gastric biopsy specimens exhibiting gastritis. In 1983 Warren and Marshall (Lancet, 1983, 1, 1273-5) reported the growth of a curved bacillus from biopsy specimens taken at gastroscopy. As this new organism was first isolated from the pylorus region of the stomach, and was campylobacter-like, it was named Campylobacter pyloridis. This was subsequently changed to the classically correct form of C.pylori.

These, or similar bacteria have been observed on the mucosa of resected stomachs of man, in association with carcinoma and ulceration, since the turn of the century. Although these organisms had been seen and reported for nearly 100 years, they were not recognised because of culture failure. This was due, either to the fastidious nature of C.pylori, or else misinterpreted as for example Pseudomonas species. It is only possible to isolate this organism from gastric biopsy material and even then critical growth conditions are needed. It has now been shown that these organisms are peculiarly adapted to their niche in gastric mucus. Their spiral morphology and high motility are

advantages in such a viscous environment. C.pylori is localised close to intercellular junctions taking advantage of the rich source of nutrients found there.

Prolonged incubation (at least three days) in a microaerobic atmosphere (6% oxygen) is required for visible colonies to be produced. Blood or serum is necessary for growth and humidity should be high.

Successful culture of this organism prompted prevalence studies, correlating endoscopic and histologic appearance, patient demography and past medical history to attempt to elucidate the pathogenic or opportunistic role of C.pylori.

Most workers now agree that the correlation between histological confirmed chronic active type B gastritis and the presence of C.pylori is more than 90 per cent. However, the correlation with duodenal ulceration is not as convincing, and workers have differed in their findings from 60 to 100 per cent. This discrepancy may be explained by the patchy distribution of gastritis or to inadequate culture techniques.

Patients colonised with C.pylori have been shown to elicit a specific immune response. A simple, highly

sensitive and specific test is needed which will reliably predict the presence or absence of these organisms, obviating the need for expensive, invasive, endoscopic procedures. The serological response to C. pylori of people attending gastroscopy and "normals" has been studied, mainly by the relatively insensitive complement fixation test (CFT).

Current tests are based on very crude antigens, which give sensitivity and specificities of between 70 and 90%, and cross react with other members of the campylobacter genus. A soluble antigen preparation used by Rathbone et al (Proceedings of the IV Campylobacter Workshop, Sweden, 1987) has improved sensitivity and specificity to around 90%. Newell et al (Serodiagn. Immunother, 1987, 1, 209-17) have modified McCoy's acid extract; this preparation has a similar protein profile to Rathbone's soluble antigen. There are reduced amounts of the 54 KDa protein so that cross reactions with other campylobacters are reduced, but not eliminated. Seven out of ten sera from patients with C. jejuni infection still produced a high titre with Newell's acid extract.

Goodwin et al (J. Inf. Dis., 1987, 155, 488-93) also used an acid extract in an ELISA test. With a cut-off value of 300 ELISA Units (EU) they attained a sensitivity of 81% and a specificity of 97%; at 150 EU, a sensitivity of 99% and specificity of 78%. However, Goodwin concluded that

-5-

"patients with intermediate results" i.e. between 150 and 300 EU "will need an endoscopic examination to determine whether C.pylori is present"; about 30% of patients were in this intermediate group.

Serology might also help to elucidate the relationship between these campylobacters and gastritis, and peptic and duodenal ulceration. This non-invasive diagnostic test lends itself to the investigation of people other than those attending gastroscopy clinics.

It has now been found that a serological test using a reagent comprising extracted Campylobacter pylori urease enzyme attached to a solid surface can very accurately determine the presence or absence of C.pylori. Such a diagnostic test has the tremendous advantage that it obviates the need for the expensive, invasive and unpleasant endoscopic examination of patients.

According to one aspect of the invention there is provided a reagent for use in the diagnosis of Campylobacter pylori infections comprising extracted C.pylori urease attached to a solid surface.

C.pylori may be isolated from human gastric mucosa or gastric epithelium (see Marshall B.J. et al, Microbios.

lett., 1984, 25, 83-88, & Kasper & Dickgiesser, Infection, 1984, 12, 179-180). Several strains of C.pylori are deposited with the National Collection of Type Cultures (NCTC) and available to anyone on request. Once isolated the bacteria can be cultered on media containing blood or serum, for 3-5 days in a microaerobic atmosphere (6% oxygen). The colonial morphology is very distinctive; the colonies are small, translucent and convex.

The C.pylori urease used for the reagent may be extracted from C.pylori by first culturing the organism on a large number of culture plates such as blood agar. Following 4 days incubation the bacteria are harvested from the plates into pH 7 phosphate buffered saline. The suspension is then extruded through a French Pressure cell and subsequently centrifuged. The C.pylori urease can be found in the suspension as demonstrated by polyacrylamide gel electrophoresis and subsequent staining of the gel with Fishbein's catalytic stain for urease (5th International Symposium on Chromotography, 1969). Urease is seen as a blue band.

The urease thus prepared can be then attached to a solid surface suitable for the diagnostic test selected. For example if the urease preparation is to be used in an ELISA test, as described by Voller in Enzyme Immunoassay, Edward T. Maggio, CRC press, p181-196, then it is attached

to microplates using passive adsorption. Such plates are usually made from plastics such as polystyrene, polyvinyl or polypropylene. Alternatively, the urease preparation could be attached to latex beads for use in a passive latex agglutination test (see Hecharry & Michaelson, Lab. Manage., 1984, 22 (6), 27-40). Attachment to the beads may be by passive adsorption, or alternatively bridging chemicals such as carbodimides, cyanogen bromide or glutaraldehyde may be used.

In an ELISA test for C.pylori according to another aspect of the present invention, serum samples from patients are added to the reagent described above, wherein the solid surface is a microplate, and incubated. Following incubation and washing, a preparation of anti-human antibody conjugated to a suitable enzyme such as B-galactosidase, peroxidase, alkaline phosphatase or glucose oxidase, is added to the solid surface and again incubated. Following incubation and washing, a substrate, usually chromogenic is added in order to assay the bound antibody-enzyme conjugate. Such substrates are initially colourless but yield a coloured product upon enzyme degradation. For peroxidase conjugates hydrogen peroxide with orthophenylenediamine can be used; para-nitrophenyl phosphate may be used for alkaline phosphatase. Therefore the presence of antibodies to C.pylori in the test sample, is determined by reading the optical density at the

-8-

appropriate wavelength.

In a passive latex agglutination test for C.pylori according to another aspect of the present invention, the C.pylori reagent described above, wherein the solid surface is latex beads, is added to samples of the test serum and agglutination of the suspended latex beads is examined for. Agglutination of the latex beads indicates the presence of C.pylori antibodies.

Use of the reagent of the present invention in such tests as ELISA test or passive latex agglutination produces a highly sensitive and specific non-invasive test which can accurately predict the presence or absence of C.pylori. The test is more precise than either culture or histological examination and eliminates the cross reactions with other campylobacter species attained with other antigenic preparations.

Urease was prepared for other urease producing pathogens, that is Staphylococcus aureus, Proteus mirabilis, Morganella morganii, Klebsiella pneumoniae and Pseudomonas aeruginosa. C.pylori positive and negative test sera from patients were cross adsorbed with each of these urease preparations, and the antibody titres to C.pylori urease, as well as to the homologous antigen, were studied. Patients with C.jejuni were also studied.

-9-

No cross reaction; were observed to the other urease preparations or to C.jejuni.

A test kit for carrying out an ELISA test may comprise the following components; microplates having the C.pylori urease attached thereto; a container of blocking buffer, which can be a simple phosphate buffer at pH 7 with 1% bovine serum albumin (BSA); a container of buffer for diluting samples; a container of anti-human antibody conjugated to an enzyme; a container of substrate for the

enzyme together with a container of diluent; a container of 3N NaOH; a container of washing solution together with instructions for carrying out the test.

The invention will be described further with reference to the following examples.

Examples

Example 1

ELISA test for the detection of C.pylori antibodies.

A recent clinical isolate of C.pylori was identified by its characteristic rapid urease reaction and Gram stain appearance. This was subcultured onto 10 blood agar plates and incubated for 4 days at 37°C in a microaerobic atmosphere. Once C.pylori had formed colonies on the plates it was harvested into phosphate buffered saline (PBS) and then washed three times in PBS. It was resuspended in 10 ml of PBS and this was extruded through a French Pressure cell at 20,000 lb/sq in. The lysate was then centrifuged at 25,000 g for 30 mins at 4°C. A Lowry protein estimation was carried out on the supernatant which contained C.pylori-specific urease.

The supernatant was then diluted with coupling buffer (a simple carbonate buffer at pH 9.6) to give a protein concentration of 10 mg/l, and 100 ul of this was then

added to each well of an ELISA plate and left overnight at 4°C. The next day the plate was blocked with 150 ul/ well of PBS/tween/0.5% bovine serum albumin (BSA) to reduce non-specific binding. This was incubated for 1 h at 25 °C and then washed four times with PBS/tween. To each well was added 100 ul of the test serum samples. The test serum is diluted with PBS/tween/1% BSA 1:200 for the detection of IgG antibodies. The plate was then incubated for 1 h at 25 °C after which it was washed with PBS/tween four times. After washing, an alkaline phosphatase conjugate anti-human antibody (100 ul/well was added to each well), the conjugate was diluted 1:800 with dilution buffer, and the plate was incubated again for two hours at 25°C. Following incubation the plate was washed four times with PBS/tween. P-Nitrophenylphosphate in diethanolamine buffer (1mg/ml) was then added to each well (100 ul/well) and the plate was incubated for 30-40 mins at 25°C. The reaction was then stopped with 3N sodium hydroxide (50 ul/well) and the optical density of each well was read at 405nm using an ELISA reader. Appropriate controls were included on each plate.

Discussion of results

The test described above was used to study 202 patients attending gastroscopy. Their C.pylori status was evaluated by microscopy of histological sections and Gram

-12-

stained tissue smears, by culture, and by a urease test on biopsy samples. A very high discrimination was found between C.pylori negative and positive patients on microbiological testing when the urease ELISA was used on serum (Figure). At a cut-off 500 optical density the new test gave no false negative results (sensitivity 100%). Furthermore 18 sera from C.pylori positive patients which had been negative on an ELISA with a soluble antigen gave high titres with the urease ELISA. There were 29 patients who had high titres in the urease assay in whom C.pylori was not detected by microbiology, 11 of these had C.pylori seen on histological examination (specificity 82%); 23 of the 29 had active gastritis, and a further 5 had intestinal metaplasia. The 12 patients with active chronic gastritis may have had C.pylori infection not detectable by histological or microbiological techniques, 3 of these had biopsy specimens taken which were inadequate for histological examination. C.pylori will only colonise gastric type tissue and so it is not surprising that the organism was not detected in the specimens with intestinal metaplasia; C.pylori has a patchy distribution and might have been present elsewhere in the stomach. There were no cross reactions with C.jejuni; 5 sera from patients infected with C.jejuni were negative. Low specificity may be explicable by C.pylori associated gastritis not detected because of sampling error or the insensitivity of microscopy and culture.

-13-

There was a high correlation between the presence of proven gastritis and antibody to C.pylori urease. One hundred and ten of the 127 patients with gastritis had urease antibodies. There were seventeen patients with gastritis in who antibody was not detected; their gastritis may have been due to another cause such as treatment with non-steriodal anti-inflammatory agents, or the recently described lymphocytic gastritis (seen in four of these patients). There were ten patients with normal histology and urease antibody; one patient had C.pylori infection, five had intestinal metaplasia.

Example 2

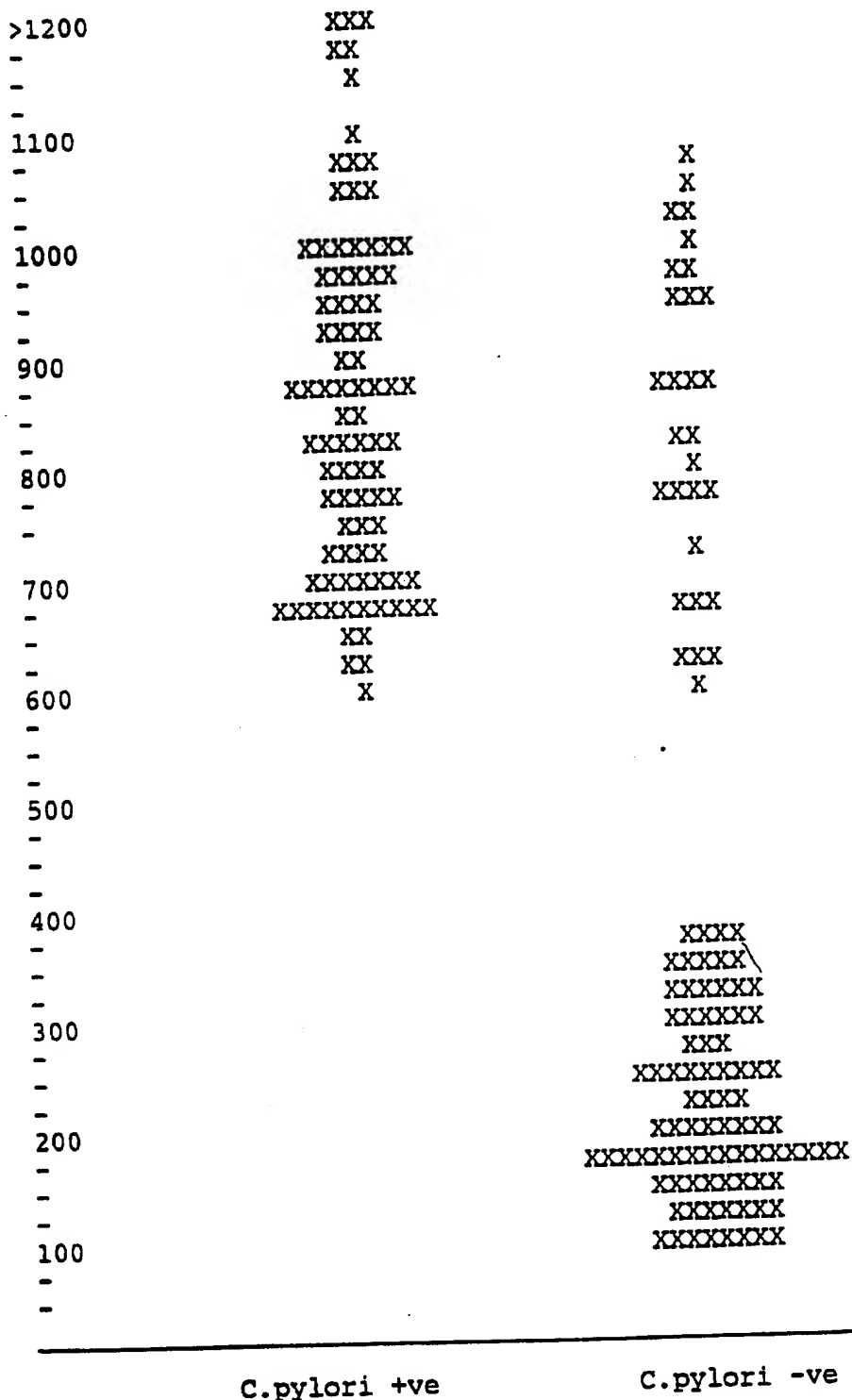
Passive latex agglutination test for the detection of C.pylori antibodies

Latex particles obtained from Sigma chemicals are sensitised (see Sherarchi at al, J. Cli. Microbiol, 26, (5), 1988, 954-956) with C.pylori specific urease extracted and prepared as described above in example 1. Following checkerboard titrations to establish optimum concentrations of antigen and test sera, sensitised latex is added to the test serum and agglutination is examined for. Appropriate controls are included and the results are expressed as either positive or negative.

LEGEND TO FIGURE

Figure 1

Absorbance values of antibody to C.pylori urease in patients from whom gastric biopsy specimens had been examined microbiologically for the presence of C.pylori.

Figure 1

CLAIMS

1. A reagent for use in the diagnosis of Campylobacter pylori infections comprising C.pylori urease attached to a solid surface.
2. A reagent as claimed in Claim 1 wherein the solid surface is a microplate.
3. A reagent as claimed in Claim 2 wherein the microplate is made from plastics such as polystyrene, polyvinyl or polypropylene.
4. A reagent as claimed in Claim 1 wherein the solid surface is latex beads.
5. A reagent for use in the diagnosis of Campylobacter pylori infections substantially as hereinbefore described and with reference to either one of the Examples.
6. A method of detecting antibodies to C.pylori in a serum sample comprising
 - i) adding the serum sample to the reagent as claimed in any one of Claims 1 to 3 to produce a test sample;
 - ii) incubating the test sample, followed by washing thereof;
 - iii) adding a preparation of anti-human antibody conjugated to an enzyme to the test sample;
 - iv) incubating the test sample, followed by washing thereof;
 - v) adding a substrate for the enzyme to the test sample;

- vi) determining the presence or absence of antibody to C.pylori in the test sample.

7. A method as claimed in Claim 6 wherein the enzyme is selected from β -galactosidase, peroxidase, alkaline phosphatase and glucose oxidase.

8. A method as claimed in Claim 6 or Claim 7 wherein the presence or absence of antibody is determined by reading the optical density of the test sample at the appropriate wavelength.

9. A method of detecting antibodies to C.pylori in a serum sample wherein the reagent as claimed in Claim 4 is added to a serum sample, incubated and examined for the presence or absence of agglutination. .

10. A method of detecting antibodies to C.pylori in a serum sample substantially as hereinbefore described with reference to either one of the Examples.

11. A test kit for use in the diagnosis of C.pylori infections comprising

- i) a reagent as claimed in any one of Claims 1 to 3;
- ii) a container of anti-human antibody conjugated to an enzyme;
- iii) a container of substrate for the enzyme; together with instructions for carrying out the test.

12. A test kit as claimed in Claim 11 wherein one or more additional containers are provided.

13. A test kit as claimed in Claim 12 wherein the additional container contains a reagent selected from blocking buffer, buffer, diluent, washing solution and sodium hydroxide.

14. A test kit substantially as hereinbefore described.

INTERNATIONAL SEARCH REPORT

International Application No PCT/GB 89/00104

I. CLASSIFICATION OF SUBJECT MATTER (If several classification symbols apply, indicate all) ⁶ According to International Patent Classification (IPC) or to both National Classification and IPC IPC4: G 01 N 33/569, 33/573; C 12 Q 1/58																	
II. FIELDS SEARCHED <div style="text-align: center; border-top: 1px solid black; border-bottom: 1px solid black;">Minimum Documentation Searched ⁷</div> <table style="width: 100%; border-collapse: collapse;"> <tr> <th style="width: 20%; border-bottom: 1px solid black;">Classification System</th> <th style="border-bottom: 1px solid black;">Classification Symbols</th> </tr> <tr> <td style="padding: 5px;">IPC4</td> <td style="padding: 5px;">G 01 N; C 12 Q</td> </tr> </table> <div style="text-align: center; border-top: 1px solid black; border-bottom: 1px solid black;">Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched ⁸</div>			Classification System	Classification Symbols	IPC4	G 01 N; C 12 Q											
Classification System	Classification Symbols																
IPC4	G 01 N; C 12 Q																
III. DOCUMENTS CONSIDERED TO BE RELEVANT ⁹ <table style="width: 100%; border-collapse: collapse;"> <tr> <th style="width: 10%; border-bottom: 1px solid black;">Category ⁹</th> <th style="width: 70%; border-bottom: 1px solid black;">Citation of Document, ¹¹ with indication, where appropriate, of the relevant passages ¹²</th> <th style="width: 20%; border-bottom: 1px solid black;">Relevant to Claim No. ¹³</th> </tr> <tr> <td style="text-align: center; vertical-align: top; padding: 5px;">A</td> <td style="padding: 5px;">WO, A1, 87/01119 (G.M. WINN) 26 February 1987, see claim 6 --</td> <td style="text-align: center; vertical-align: top; padding: 5px;">1, 6, 11</td> </tr> <tr> <td style="text-align: center; vertical-align: top; padding: 5px;">P, A</td> <td style="padding: 5px;">J. Med. Microbiol., Vol. 27, 1988 R.L. Ferrero et al.: "The urease enzymes of Campylobacter pylori and a related bacterium", see page 33 - page 40 see page 39, right column --</td> <td style="text-align: center; vertical-align: top; padding: 5px;">6</td> </tr> <tr> <td style="text-align: center; vertical-align: top; padding: 5px;">A</td> <td style="padding: 5px;">SCAND. J. GASTROENTEROLOGY, Vol: 23, SUPPL. 142, 1988, Proc. Intern. Workshop Copenhagen, 15 und 16 October 1987, F. Mégraud: "Comparison of Different Tests for Campylobacter pylori", see page 58 - page 68 see page 64, left column</td> <td style="text-align: center; vertical-align: top; padding: 5px;">6</td> </tr> <tr> <td style="text-align: center; vertical-align: top; padding: 5px;">A</td> <td style="padding: 5px;">D.G. Newell et al: "An Enzyme-linked Immuno-sorbent Assay for the Serodiagnosis of Campylobacter pylori-associated Gastritis", see page 53 - page 57 -----</td> <td style="text-align: center; vertical-align: top; padding: 5px;">1-3, 5-8 10-14</td> </tr> </table>			Category ⁹	Citation of Document, ¹¹ with indication, where appropriate, of the relevant passages ¹²	Relevant to Claim No. ¹³	A	WO, A1, 87/01119 (G.M. WINN) 26 February 1987, see claim 6 --	1, 6, 11	P, A	J. Med. Microbiol., Vol. 27, 1988 R.L. Ferrero et al.: "The urease enzymes of Campylobacter pylori and a related bacterium", see page 33 - page 40 see page 39, right column --	6	A	SCAND. J. GASTROENTEROLOGY, Vol: 23, SUPPL. 142, 1988, Proc. Intern. Workshop Copenhagen, 15 und 16 October 1987, F. Mégraud: "Comparison of Different Tests for Campylobacter pylori", see page 58 - page 68 see page 64, left column	6	A	D.G. Newell et al: "An Enzyme-linked Immuno-sorbent Assay for the Serodiagnosis of Campylobacter pylori-associated Gastritis", see page 53 - page 57 -----	1-3, 5-8 10-14
Category ⁹	Citation of Document, ¹¹ with indication, where appropriate, of the relevant passages ¹²	Relevant to Claim No. ¹³															
A	WO, A1, 87/01119 (G.M. WINN) 26 February 1987, see claim 6 --	1, 6, 11															
P, A	J. Med. Microbiol., Vol. 27, 1988 R.L. Ferrero et al.: "The urease enzymes of Campylobacter pylori and a related bacterium", see page 33 - page 40 see page 39, right column --	6															
A	SCAND. J. GASTROENTEROLOGY, Vol: 23, SUPPL. 142, 1988, Proc. Intern. Workshop Copenhagen, 15 und 16 October 1987, F. Mégraud: "Comparison of Different Tests for Campylobacter pylori", see page 58 - page 68 see page 64, left column	6															
A	D.G. Newell et al: "An Enzyme-linked Immuno-sorbent Assay for the Serodiagnosis of Campylobacter pylori-associated Gastritis", see page 53 - page 57 -----	1-3, 5-8 10-14															
<div style="display: flex; justify-content: space-between;"> <div style="width: 45%;"> <p>¹⁰ Special categories of cited documents: ¹⁰</p> <p>"A" document defining the general state of the art which is not considered to be of particular relevance</p> <p>"E" earlier document but published on or after the international filing date</p> <p>"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)</p> <p>"O" document referring to an oral disclosure, use, exhibition or other means</p> <p>"P" document published prior to the international filing date but later than the priority date claimed</p> </div> <div style="width: 45%;"> <p>"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention</p> <p>"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step</p> <p>"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.</p> <p>"A" document member of the same patent family</p> </div> </div>																	
IV. CERTIFICATION <table style="width: 100%; border-collapse: collapse;"> <tr> <td style="width: 50%; border-bottom: 1px solid black; padding: 5px;"> Date of the Actual Completion of the International Search 21st April 1989 </td> <td style="width: 50%; border-bottom: 1px solid black; padding: 5px;"> Date of Mailing of this International Search Report <div style="text-align: center; font-weight: bold;">17 MAY 1989</div> </td> </tr> <tr> <td style="border-bottom: 1px solid black; padding: 5px;"> International Searching Authority <div style="text-align: center; font-weight: bold;">EUROPEAN PATENT OFFICE</div> </td> <td style="border-bottom: 1px solid black; padding: 5px;"> Signature of Authorized Officer <div style="text-align: center;"> P.C.G. VAN DER PUTTEN </div> </td> </tr> </table>			Date of the Actual Completion of the International Search 21st April 1989	Date of Mailing of this International Search Report <div style="text-align: center; font-weight: bold;">17 MAY 1989</div>	International Searching Authority <div style="text-align: center; font-weight: bold;">EUROPEAN PATENT OFFICE</div>	Signature of Authorized Officer <div style="text-align: center;"> P.C.G. VAN DER PUTTEN </div>											
Date of the Actual Completion of the International Search 21st April 1989	Date of Mailing of this International Search Report <div style="text-align: center; font-weight: bold;">17 MAY 1989</div>																
International Searching Authority <div style="text-align: center; font-weight: bold;">EUROPEAN PATENT OFFICE</div>	Signature of Authorized Officer <div style="text-align: center;"> P.C.G. VAN DER PUTTEN </div>																

ANNEX TO THE INTERNATIONAL SEARCH REPORT
ON INTERNATIONAL PATENT APPLICATION NO. PCT/GB 89/00104

PCT/GB 89/00104

SA 26730

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report. The members are as contained in the European Patent Office EPO file on 03/03/89. The European Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO-A1- 87/01119	26/02/87	AU-D- EP-A-	62827/86 0233261
			10/03/87 26/08/87

For more details about this annex : see Official Journal of the European Patent Office, No. 12/82